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Scanning-Beam Electron Microscopy of Cell Wall-Defective Staphylococci

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Staphylococci and their derived wall-defective forms (WDS) were studied with Gram stain, phase microscopy, and the scanning-beam electron microscope. Staphylococci were smooth, spherical, and relatively uniform in size. Stable WDS had corrugated surfaces and were larger; those prepared with lysostaphin were indistinguishable from those prepared with methicillin. During induction of WDS in methicillin-containing hypertonic broth, WDS were first observed after 7 hr of incubation and progressively, thereafter, increased in number. They were larger than the stable WDS and varied more in size and shape. Microscopically, "wisps" were seen to consist of WDS, persistent parent staphylococci, and residual cell membranes.

The scanning-beam electron microscope (SEM), although extensively employed in industrial research, has only recently been utilized in biological investigations. For the study of microorganisms, its advantages over the conventional transmission electron microscope are that it allows study of the surfaces of cells at high magnification in 3-dimensional perspective and permits visualization of large populations of cells.

During the past few years, the Infectious Disease Division of the University of Cincinnati College of Medicine has investigated various aspects of wall-defective staphylococci (1, 2, 4, 5). The term wall-defective staphylococci (WDS) is employed in this paper to designate cell wall-defective forms prepared either by growth in methicillin-containing broth or by dissolution of cell wall by the muralytic enzyme lyostaphin. Except for the stable forms, where absence of hexosamine has been shown biochemically, the presence or absence of minute amounts of wall materials was not known (4). Meanwhile, at the Ohio State University College of Medicine, the Division of Infectious Diseases has studied normal staphylococci as seen with the SEM (3). These experiences provided an unusual opportunity for a collaborative investigation. In the present study, we have compared the appearance of staphylococci with that of stable WDS prepared either by growth in methicillin-containing broth or by dissolution of cell walls by a muralytic enzyme (lysostaphin). We have likewise observed staphylococci at various stages during their conversion to the wall-defective forms in hypertonic broth containing methicillin. To our knowledge, this is the first demonstration of the surface morphology of intact WDS.

MATERIALS AND METHODS

Microorganisms. The August Harmon strain of *Staphylococcus aureus*, originally isolated from a patient with endocarditis, was utilized for all experiments. Its phage type is 6/7/42D/54/75. With an inoculum of 10^6 cells per ml, the minimum inhibiting concentration of penicillin G is 1,500 μg/ml, and for methicillin, 3.12 μg/ml. Stable WDS of this strain induced with methicillin (1) or lysostaphin (5) were also used; the former had been subcultured more than 300 times and the latter more than 150 times in hypertonic broth free of the respective inducing agents. These stable forms have been shown to be free of detectable cell wall material (4).

Culture media. Brain Heart Infusion Broth (BHI, Difco) was the medium employed in all of the experiments. To provide osmotic stability for the WDS, the osmolarity was increased from 400 to 2,500 milliosmols per liter by raising the NaCl concentration from 0.5% to 5% and by adding 0.2% $MgSO_4$; this medium will be referred to as salt broth. In some experiments, 5% human serum, inactivated at 56 C for 30 min, was added to salt broth; this medium will be referred to as serum-salt broth.

Subcultures for colony counts of the parent staphy-

lococci and WDS were made as previously described; typical "fried egg" colonies (L colonies) were recognized as originating from the WDS (1).

Preparation of specimens for SEM. Cultures of the parent staphylococci and WDS were harvested by centrifugation at 450 × g for 15 min, washed in 10 ml of 5% saline five times, and fixed in 0.25% buffered glutaraldehyde (*p*H 7.2) for a minimum of 2 hr at room temperature on a rotating drum. They were then washed five times with, and resuspended in, 2 ml of demineralized distilled water which had been passed through a membrane filter (pore size: 0.45 μm; Millipore Corp., Bedford, Mass.) and then autoclaved. A drop of each suspension was placed on a 5-mm disc of heavy-duty aluminum foil (Reynolds Wrap Food Service Foil, Reynolds Metals Co., Richmond, Va.) and allowed to dry at room temperature overnight. The discs were then attached to specimen stubs with aquacol (Dag-dispersion 154, Acheson Colloids Co., Port Huron, Mich.). Specimens were coated with pure gold on a rotary turntable high-vacuum coating unit (Varian Vacuum Evaporator VE10, Ernest F. Fullam, Inc., Schenectady, N.Y.) to obtain a uniform coating approximately 10 nm thick.

SEM. A Cambridge Stereoscan Electron Microscope (Mark II, Cambridge Instrument Co., Ltd., London, England) was employed. The specimen stage was adjusted so that the electron beam would strike it at a 22 to 31° angle. Specimens were examined and photographed at accelerating voltages of 20 and 30 kv. A scan time of 100 sec was employed, utilizing 1,000 lines per 4-inch frame. Photographs were obtained with Polaroid Sheet film (type 55 P/N, Polaroid Corp., Cambridge, Mass.).

RESULTS

Surface appearance of staphylococci and their derived stable wall-defective forms. The parent staphylococci were cultured in BHI broth and in salt broth; stable WDS were cultured in salt broth. Samples were harvested after various periods of incubation, ranging from 4 hr to 7 days, and examined by Gram stain, phase microscopy, and the SEM.

Many thousands of staphylococci and WDS were seen with the SEM. The parent forms varied very slightly in size and shape and, in general, were smooth and spherical (Fig. 1A). Their appearance was monotonously uniform, regardless of the media in which they were cultured or the duration of their incubation, and resembled another strain previously reported (3).

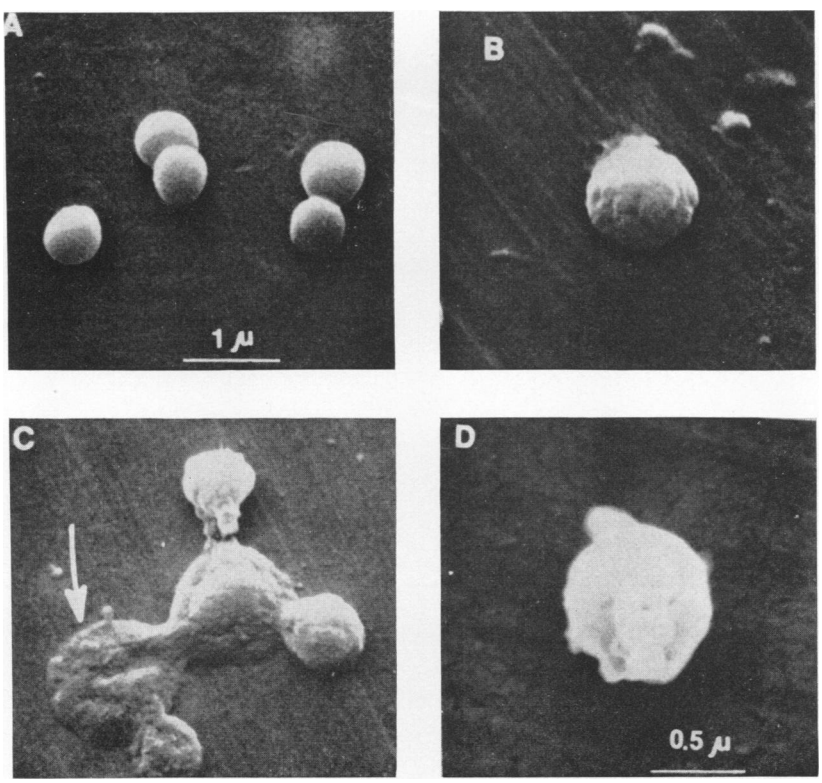

FIG. 1. *SEM photomicrograph of S. aureus and stable WDS. (A) S. aureus, × 10,000. (B) Lysostaphin-induced WDS, × 10,000. (C) Methicillin-induced WDS. Arrow shows the "ghost" of a collapsed cell, × 10,000. (D) Methicillin-induced WDS demonstrating surface blebs, × 20,000.*

The stable WDS were easily distinguishable from the parent forms; those prepared with lysostaphin (Fig. 1B) were indistinguishable from those prepared with methicillin (Fig. 1C). They were larger than the parent forms. Their surfaces were corrugated in appearance and showed variable numbers of surface "blebs" (Fig. 1D). Some were prone to collapse, leaving "ghosts" which presumably were cell membranes (Fig. 1C).

By Gram stain, the parent forms were gram-positive; the WDS were larger and gram-negative.

By phase microscopy, normal staphylococci were small, uniform in size, and smooth in appearance. Stable WDS were larger and varied in size. This variation was only moderate in young cultures, but cultures incubated 2 days or more exhibited greater variation, with increasing numbers of very large forms, more granular bodies at the periphery of individual cells, and various amounts of cellular debris.

SEM observations during induction of wall-defective staphylococci in methicillin-containing broth. Eighteen-hour cultures of *S. aureus* in BHI broth, salt broth, and serum-salt broth were used as inocula; colony counts showed that each contained approximately 7×10^8 bacteria per ml. A 10-ml amount of each was inoculated into flasks containing 90 ml of the corresponding media and methicillin (100 μg/ml). This exceptionally large inoculum was necessitated by the unavoidable loss of some cells during the washings used in preparation for SEM microscopy. A control flask of serum-salt broth without methicillin was similarly inoculated. The contents of the four flasks are shown in Table 1.

The flasks were incubated for 21 days at 37 C. Samples (2 to 5 ml, depending on turbidity) were removed after 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 11 hr and 1, 2, 3, 6, 12, and 21 days of incubation and examined by Gram stain, phase microscopy, and the SEM. Concentrated methicillin was added each day to the methicillin-containing flasks to maintain the concentration at approximately 100 μg per ml.

Inocula of staphylococci were sufficiently heavy that all flasks were initially turbid. In flask A, containing normal BHI broth and methicillin, normal staphylococci were initially seen by Gram stain, phase microscopy, and the SEM. During the first 3 days of incubation, the flask cleared and staphylococci became sparse by all methods of microscopy; with the SEM, a progressively increasing number of cells were seen to be degenerating (Fig. 2). By the second day, most of the staphylococci had degenerated, and only rare degenerative forms could be seen by the sixth day. By day 12, no organisms of any sort were seen, and subcultures from the flask showed no growth.

Flasks B and C, containing salt broth and serum-salt broth, respectively, and methicillin, showed some initial clearing but also displayed the typical "wisp-like" growth of WDS within the first day. During the first 6 hr of incubation, no WDS were seen; after 7 hr of incubation, a few WDS could first be seen by Gram stain, phase microscopy, and the SEM. With increasing periods of incubation, their relative numbers increased, so that after 2 days of incubation they could be found with ease (Fig. 3). The WDS varied considerably in size and shape (Fig. 4, 5) and were larger than the stable WDS described above. The number of parent staphylococci diminished and gram-positivity disappeared by day 12, but morphologically normal persisters were seen with the SEM and could be subcultured throughout the experiment. Eventually, however, the WDS predominated (Fig. 6, 7). WDS, persistent staphylococci, and residual cell membranes adhered to each other and formed a conglomerate which may adequately explain the structure of the wisps which were seen grossly (Fig. 8).

The staphylococci in flask D, containing serum-salt broth without methicillin, remained morphologically normal throughout the ex-

TABLE 1. *Contents of the flasks used during induction of cell wall-defective staphylococci in methicillin-containing broth*[a]

Flask	Medium	NaCl	$MgSO_4$	Serum	Methicillin	*Staphylococcus aureus*
		%	%	%	μg/ml	*per ml*
A, BHI broth	BHI	0.5	0	0	100	7×10^7
B, salt broth	BHI	5	0.2	0	100	7×10^7
C, serum-salt broth	BHI	5	0.2	5	100	7×10^7
D, serum-salt broth without methicillin	BHI	5	0.2	5	0	7×10^7

[a] Final volume in all flasks was 100 ml.

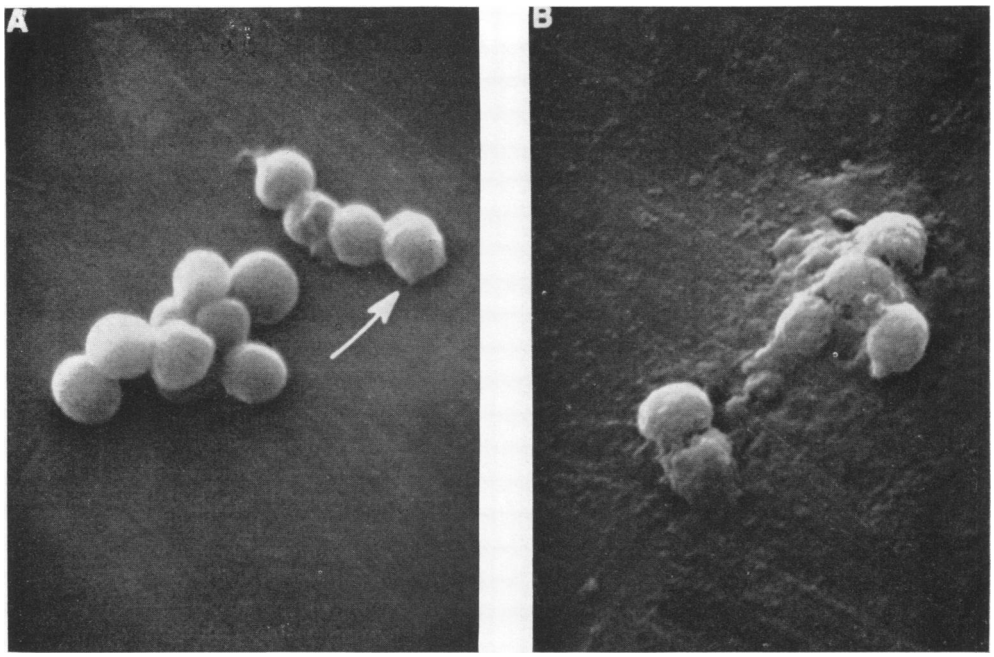

FIG. 2. *S. aureus in methicillin-containing BHI broth. (A) Occasional microorganisms (arrow) showing early degenerative changes at 5 hr. (B) By 2 days, most of the cells had degenerated. × 10,000.*

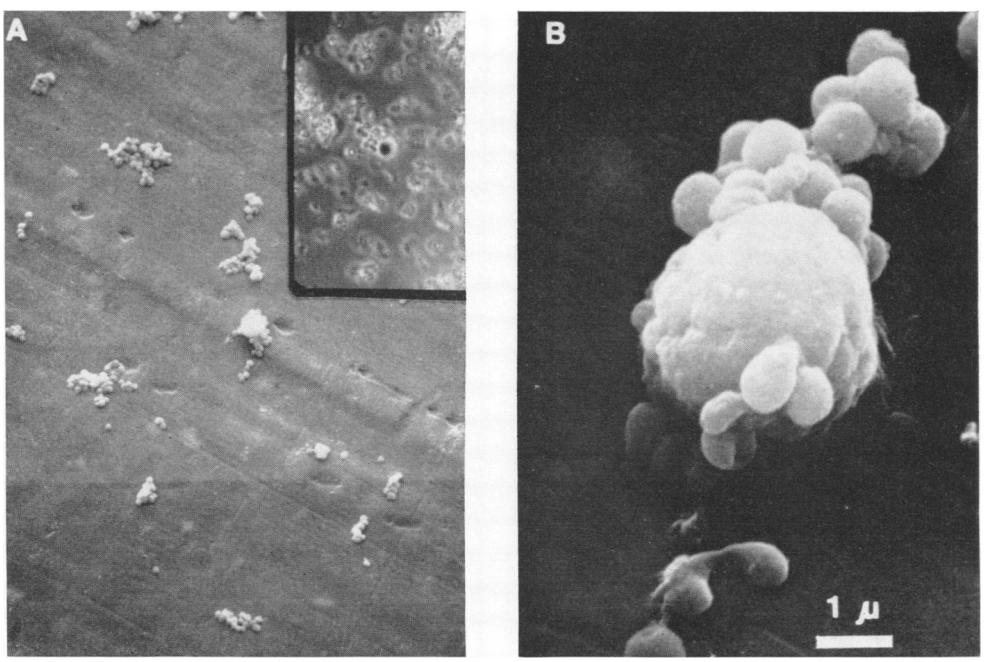

FIG. 3. *S. aureus in methicillin-containing salt broth at 2 days. Occasional WDS are easily seen. (A) × 1,000. Insert shows similar view by phase-contrast. (B) × 10,000.*

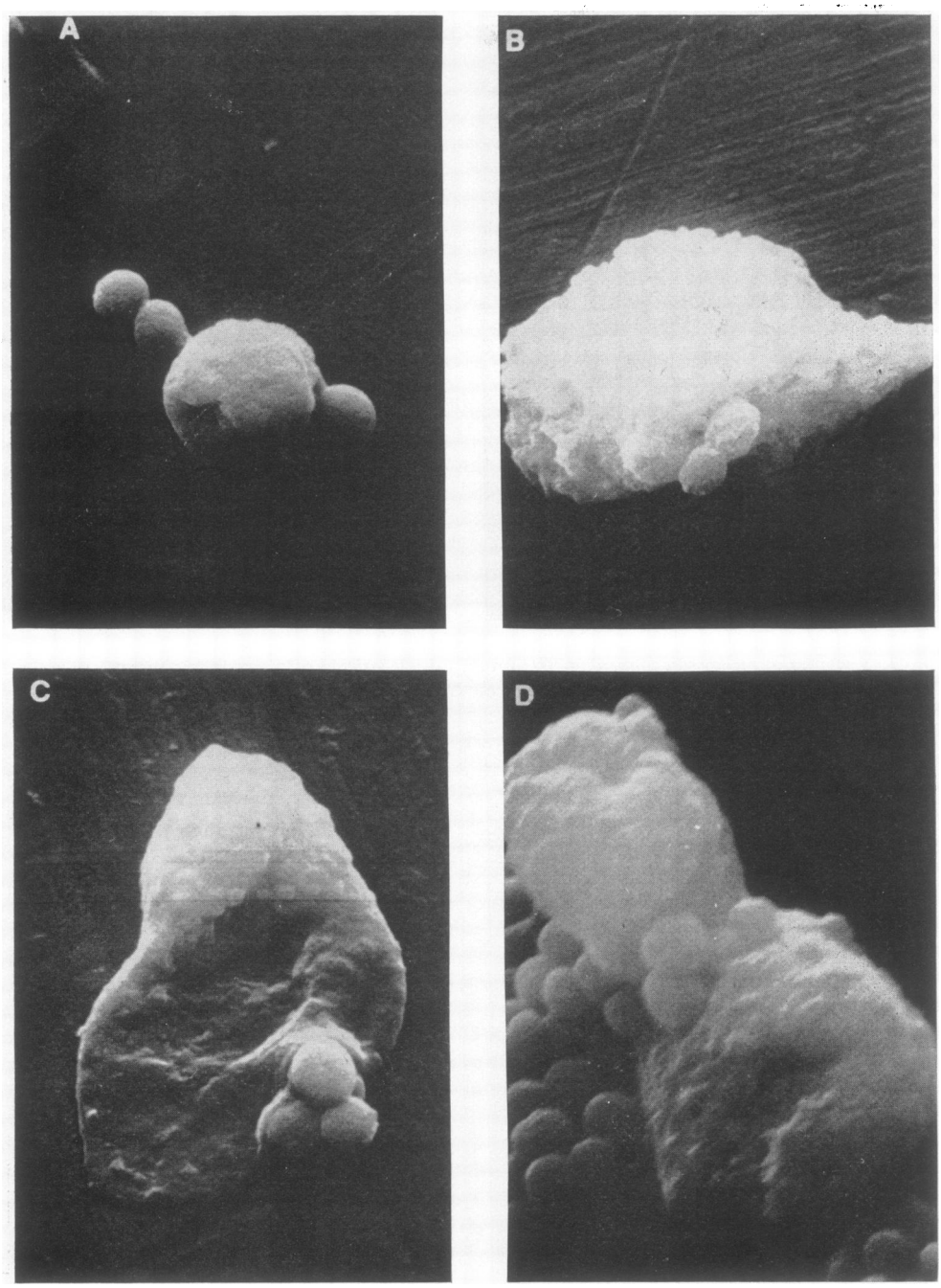

FIG. 4. *S. aureus in methicillin-containing serum-salt broth. WDS of various sizes and shapes and persistent staphylococci. (A, B, C, D) 1 to 6 days. × 10,000.*

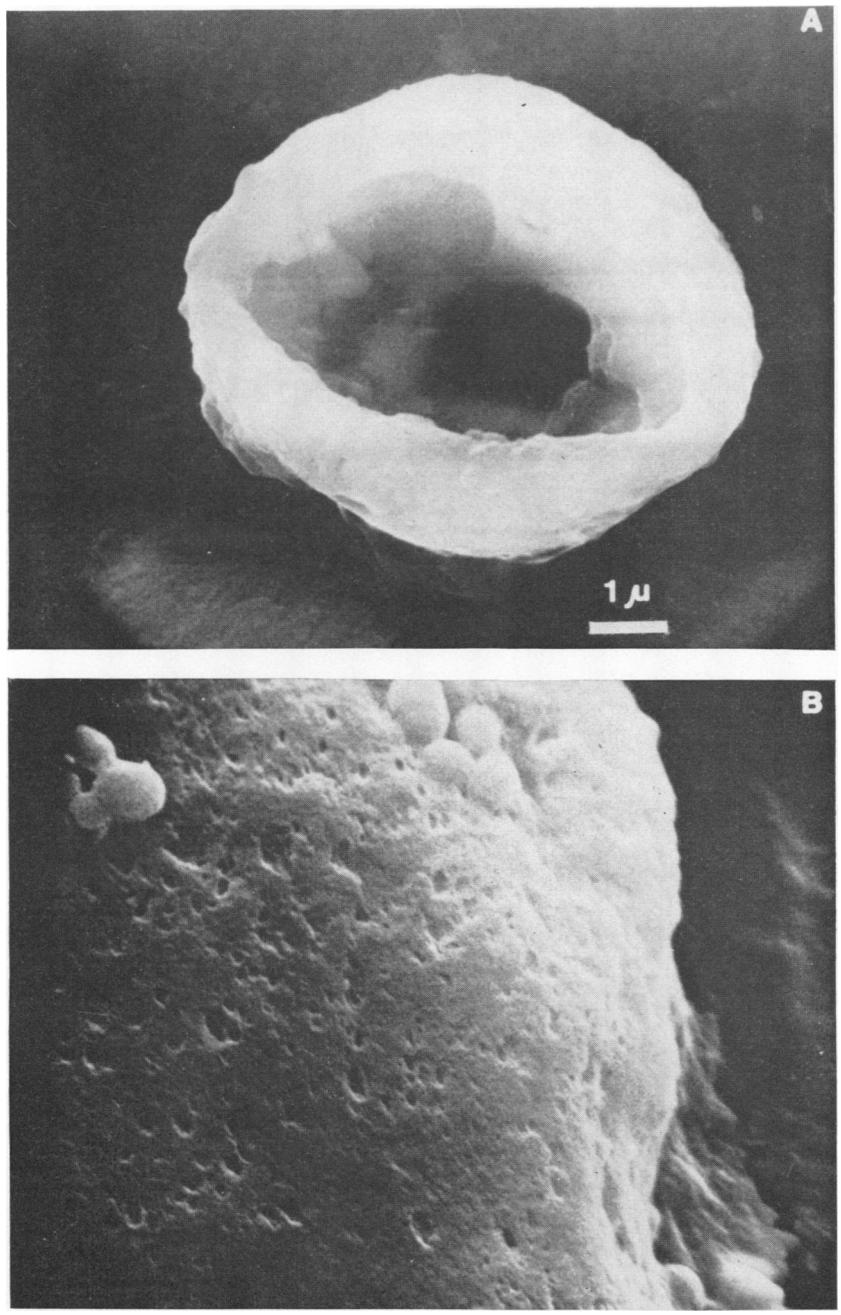

FIG. 5. *S. aureus in methicillin-containing serum-salt broth. (A) Collapsed WDS at 6 days. (B) Minute defects in surface of a WDS 30 μm in diameter at 12 days. × 10,000.*

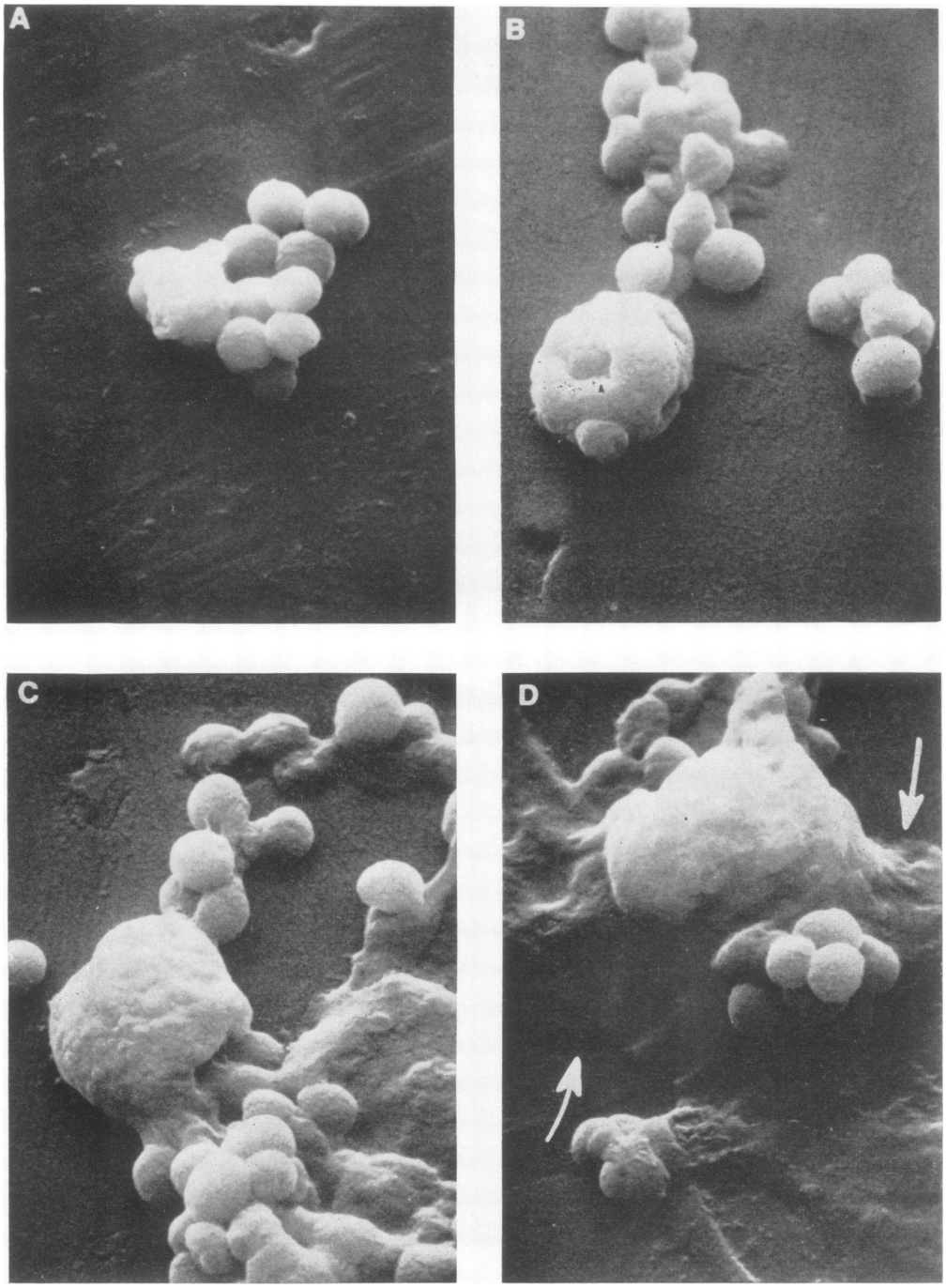

FIG. 6. *S. aureus in methicillin-containing serum-salt broth. Progressively larger WDS are seen. Collapse results in residual cell membranes (arrows). (A) 2 days. (B, C, D) 6 days. × 10,000.*

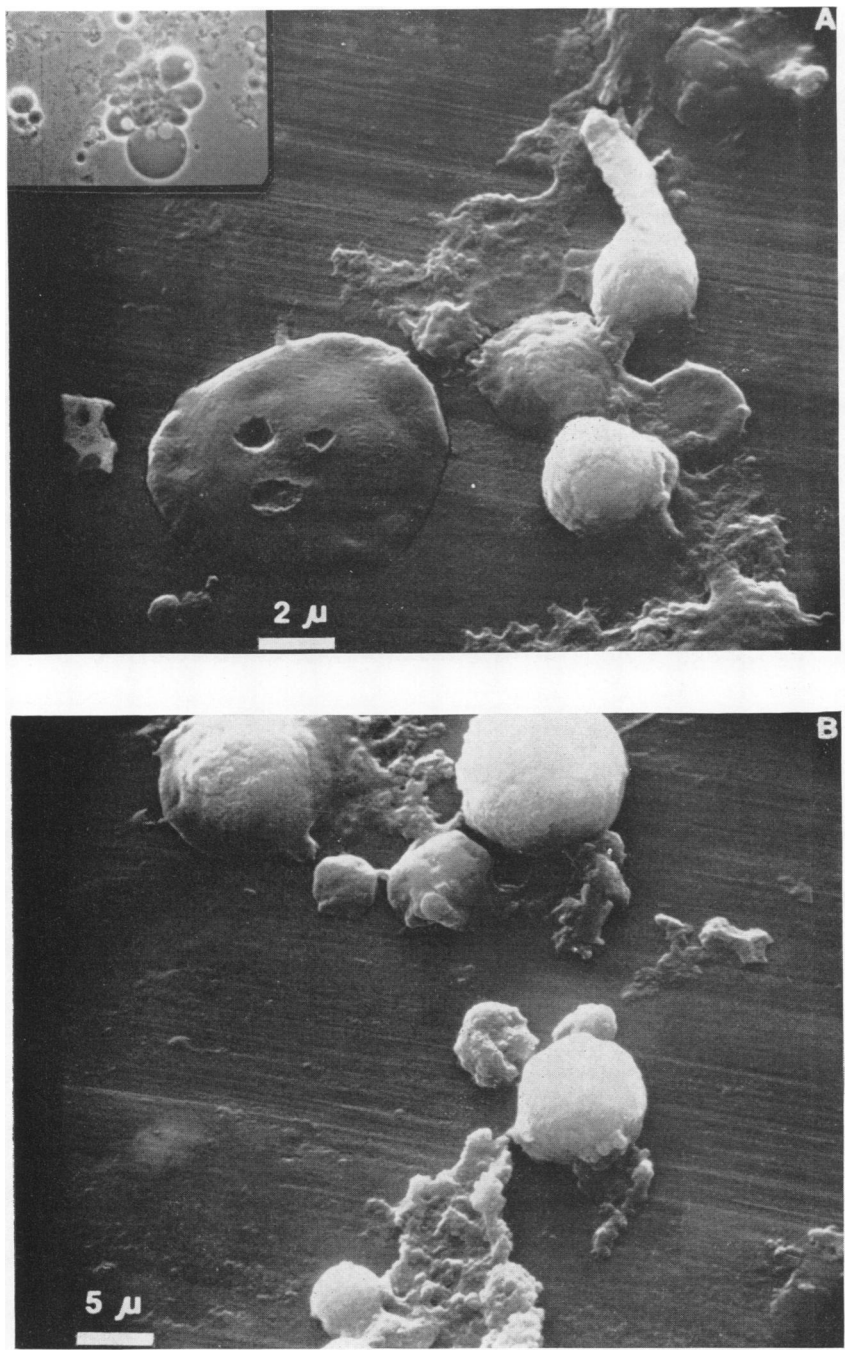

FIG. 7. *S. aureus in methicillin-containing serum-salt broth at 12 days. Many WDS of various sizes and shapes and residual cellular debris consisting of cell walls and membranes. (A) × 5000. (B) × 2000. Insert shows phase-contrast photomicrograph from the same flask at 12 days.*

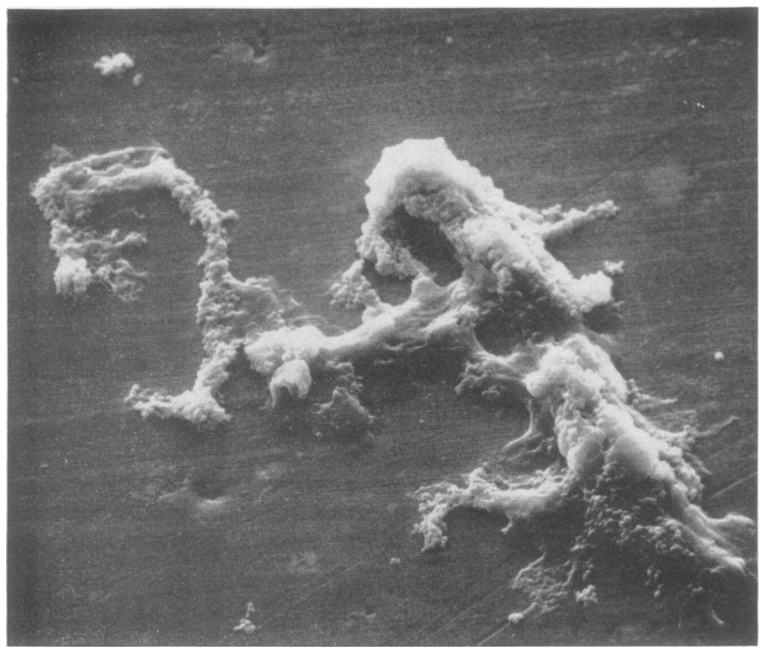

FIG. 8. *S. aureus in methicillin-containing serum-salt broth for 12 days demonstrating "wisp-like" structure of adherent WDS, persistent staphylococci, and residual cell membranes.* × *1000.*

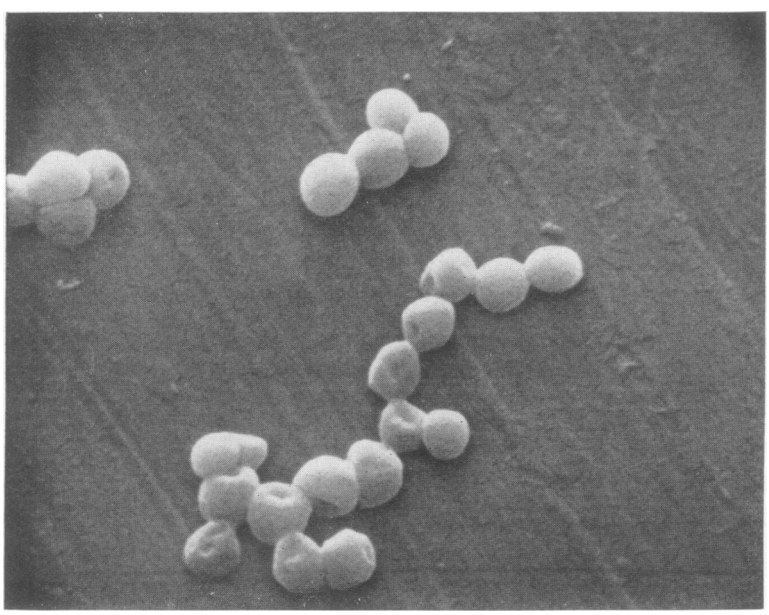

FIG. 9. *S. aureus in serum-salt broth without methicillin at 2 days. The microorganisms appear normal except for dimpling of the surface.* × *10,000.*

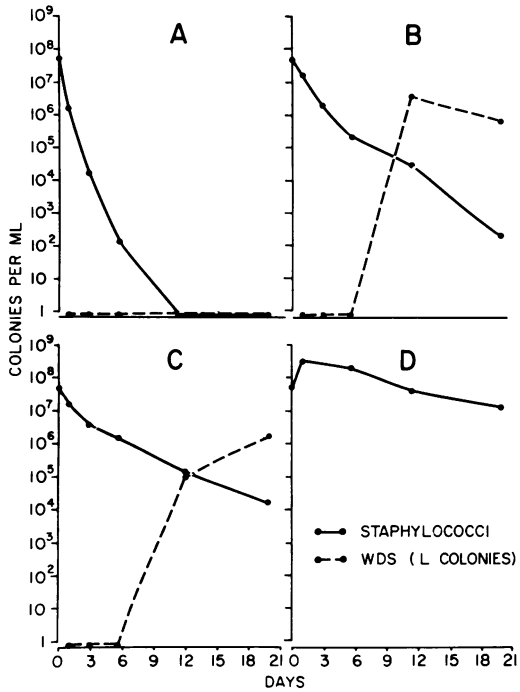

FIG. 10. *Serial colony counts of staphylococcal colonies and L colonies during incubation of S. aureus in broth. Flask A: Brain Heart Infusion Broth and methicillin. Flask B: Salt broth and methicillin. Flask C: Serum-salt broth and methicillin. Flask D: Serum-salt broth without methicillin.*

periment although some dimpling, presumably osmotic dehydration, of the cells occurred (Fig. 9).

The serial counts of parent staphylococcal colonies and L colonies are shown in Fig. 10. The progressive decrease in the number of staphylococcal colony-forming units in the presence of methicillin, the persistence of some parent forms, and the proliferation of WDS in the hypertonic media are illustrated.

Unusual forms. During the above experiments, unusual morphological forms, which may or may not be detectable by Gram stain or phase microscopy, were occasionally seen with the SEM (Fig. 11), in an unpredictable fashion. They fell into four general categories: minute spherical forms, microaggregates of WDS, rod-shaped structures, and filamentous forms.

DISCUSSION

Observations of bacteria and WDS by light microscopy are limited by the relatively low magnifications achievable; at × 1,000, fine morphological detail is not discernible. With the transmission electron microscope, ultrathin sections show cross-sectional ultrastructure at magnifications unachievable by any other method. Techniques for the study of surface morphology are cumbersome and the results give limited information about surface structure of large populations of cells. With the SEM, preparation of specimens is relatively simple and the surface morphology of large populations of bacteria and their derived wall-defective forms can be studied in true three-dimensional perspective with minimal distortion.

In the present study, WDS were clearly distinguishable from the parent forms and had a unique surface morphology which was demonstrable whether the cell wall was removed by growth in the presence of methicillin or by the lytic action of lysostaphin. The staphylococci which converted to WDS upon exposure to methicillin in hypertonic broth developed irregular surfaces and progressively enlarged; collapse and "ghost" formation were frequent phenomena. Unlike previous experiences with smaller inocula of staphylococci (1), the addition of serum was not necessary for the induction of WDS in salt broth. In isotonic media, slightly enlarged staphylococci were seen rarely, and the bacteria degenerated without passing through a demonstrable phase of enlargement; WDS formation was not observed.

The unusual forms seen with the SEM during the present study have fascinated us. Their appearance was neither predictable nor consistently reproducible, yet their presence could not be denied. Contamination did not seem to account for their presence since, each time they were seen, attempts to subculture a contaminating organism failed. Studies are underway to explain their genesis and significance.

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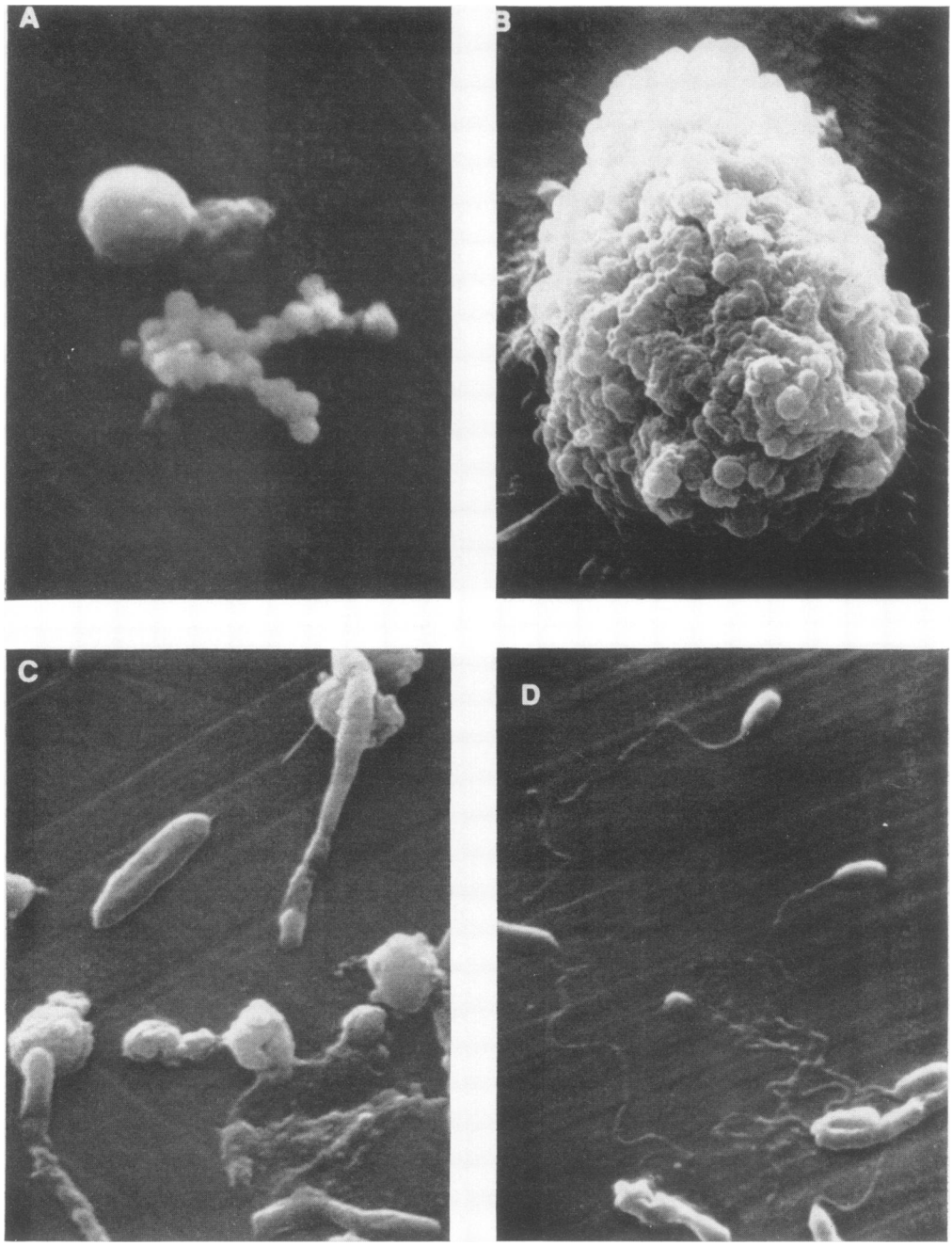

FIG. 11. *Unusual morphological forms seen with the SEM. (A) Minute spherical forms 0.2 μm in diameter and a staphylococcus in methicillin-containing BHI broth for 2 days. × 20,000. (B) Microaggregate of lysostaphin-induced stable WDS. Eighteen-hour culture. × 5,000. (C) Rod-shaped structures and lysostaphin-induced stable WDS. Eighteen hour culture. × 10,000. (D) Filamentous forms. Eighteen-hour culture of lysostaphin-induced stable WDS. × 10,000.*

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